PNA Arrays for miRNA Detection

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(Received February 20, 2009; CL-090177; E-mail: ohtsuk@cc.okayama-u.ac.jp)

The profiling of microRNAs (miRNAs) using microarray technology has been used in many recent biological and medical studies. In this study, arrays of peptide nucleic acids (PNAs) were prepared and their miRNA capture abilities were evaluated. We found that the sensitivity of 10-mer PNA probes in targeting miRNAs was much higher than that of the corresponding DNA probes and also of longer (20-mer) DNA probes.

MicroRNAs (miRNAs) are short RNAs that control gene expression at the post-transcriptional level and play important roles in various physiological processes, such as cell differentiation and development.[1] Recently, miRNA expression patterns in various tissues and cell types have been revealed by miRNA profiling studies. Specific miRNA expression patterns provide valuable diagnostic and prognostic indications for human diseases.[2] Microarrays are commonly used for global miRNA profiling, and DNA probes are generally used in most microarrays for miRNA detection.[3] However, certain nucleic acid analogs, with more favorable hybridization characteristics than DNA probes, could be more suitable for use as miRNA detection probes.

Peptide nucleic acids (PNAs) are nucleic acid analogs with an achiral polyamide backbone.[4] Since the PNA backbone is not charged, there is no charge repulsion between a PNA strand and an RNA or DNA strand, and thus PNA–RNA hybrids are thermally more stable than the corresponding DNA–RNA hybrids.[5] Locked nucleic acids (LNAs) also recognize RNA with high specificity and high affinity.[6] LNA probes are an emerging tool for miRNA detection.[7] However, there have been no reports of miRNA detection using PNA arrays except those for DNA detection,[8] although with certain sequences, a PNA–RNA hybrid was shown to be more stable than the corresponding LNA–RNA hybrid.[9]

In this study, in order to evaluate the potential of PNA probes for detecting miRNAs, we first prepared the PNA arrays. In our previous experiments on RNA isolation using PNA probes, the amount of target RNA obtained using an 8-mer PNA was lower than with a 10- or 12-mer PNA. We also found that a 12-mer PNA probe captured more nonspecific RNAs than a 10-mer PNA probe.[10] Therefore, we used 10-mer PNA probes in this study.

The PNA probes were designed to hybridize to sequences near the 3′-end of their target RNAs. The PNAs were synthesized by an Fmoc solid-phase method as described in our previous study,[10] and the PNA sequences used in this study are listed in Table 1. To improve water solubility, every PNA was modified with an ethylene glycol linker (Fmoc-21-amino-4,7,10,13,16,19-hexaheneicosanoic acid; NeoMPS) at the N-terminus, and two lysine units at the C-terminus. In addition, each PNA contained an amino group at the N-terminus. The DNAs bearing an amino-linker at the 5′-end and the miRNAs used in this study (Table 1) were purchased from Japan Bio Services Co., Ltd.

Table 1. Sequences of PNAs, DNAs, and miRNAs used in this study

PNA/DNA	Sequence
mi16–PNA	N-CGCCAATATT-C
mi16–mismatch-PNA	N-CGCCTATATT-C
mi22–PNA	N-ACAGTTCTTC-C
mi92–PNA	N-CAAGTGCAAT-C
mi16–DNA-10	5′-CGCCAATATT-3′
mi92–DNA-10	5′-CAAGTGCAAT-3′
mi16–DNA-20	5′-CGCCAATATTTACGTGCTGC-3′
mi92–DNA-20	5′-AGGCCGGGACAAGTGCAATA-3′
miRNA	Sequence
miR–16	5′-AGCAGCACGUAAAUAUUGGCG-3′
miR–92	5′-UAUUGCACUUGUCCCGGCCUG-3′

Cy5-labeling of each miRNA was performed using a mirVana™ miRNA labeling kit (Applied Biosystems) with slight modifications. Briefly, supplemental ATP (200 μM final concentration) was added to the RNA elongation reaction with miRNA (300 ng) and amine-modified nucleotides. The reaction product was purified and coupled to a Cy™5 monofunctional amine-reactive dye (GE healthcare) according to the manufacturer's protocol. The Cy5-labeled miRNA was resuspended in 12 μL of hybridization buffer [10 mM Tris-HCl (pH 7.5), 10 mM EDTA] and stored at −80 °C until use.

PNA array preparation and miRNA hybridization were performed as follows: HPLC-purified PNA, or synthetic DNA, was diluted in 150 mM phosphate buffer (pH 8.5) at various concentrations. They were then spotted onto a glass slide with aldehyde-surface (Greiner) using an SPBIO-2000 arrayer (Hitachi Software). The glass slide was incubated in a humidified Micro-Array Hybrid Chamber (Camlab) overnight at 25 °C to form a Schiff base between the glass slide and the amino group at the N-terminus of the PNA, or at the 5′-end of the DNA. After the incubation, the glass slide was washed with 0.2% SDS for 5 min, followed by two washes with distilled water for a total of 10 min. Subsequently, the unreacted aldehyde group was deactivated at room temperature for 5 min in 2.0 g/L $NaBH_4$ solution solublized in 25% ethanol/PBS, followed by 90 °C for 2 min in H_2O. The glass slide was then washed twice with 0.2% SDS for a total of 2 min and distilled water for 1 min. The slide was then rinsed with hybridization buffer and distilled water, and dried in a 50 mL centrifuge tube at 2300 × *g* for 2 min. Cy5-labeled miRNA (11 μL) was dropped onto a 24 × 22 mm Spaced Cover Glass (Takara), and layered onto the glass slide modified with the PNAs and DNAs. The slide was incubated in a humidified Micro-Array Hybrid Chamber at 50 °C for 2 h. The slide was then washed with hybridization buffer for 5 min, and centrifuged in a 50-mL centrifuge tube at 2300 × *g* for 2 min. A fluorescence image scanner FMBIO III (Hitachi) was used to detect the Cy5 signal of the hybridized miRNA.

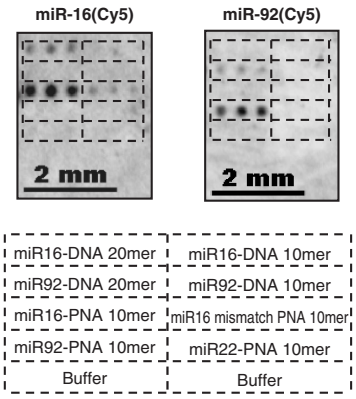

Figure 1. Detection of Cy5-labeled miR-16 (left panel) and miR-92 (right panel) on a PNA/DNA array. The top panel shows 20 μM of each PNA or DNA probe spotted onto glass slides.

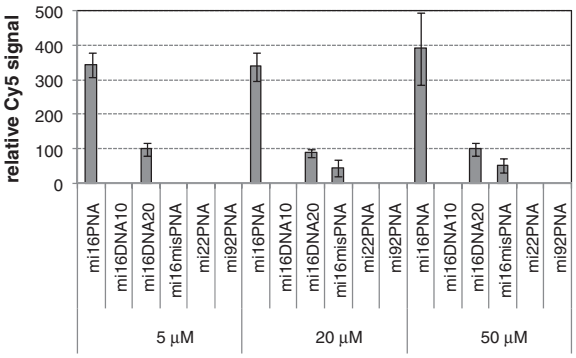

Figure 2. Fluorescent signal levels of Cy5-labeled miR-16 detected on a PNA/DNA array. The PNAs and DNAs were spotted onto glass-slides at 5, 20, and 50 μM.

In Figure 1, 20 μM of each PNA probe was spotted in a three replicate array format together with the DNA probe. The target miRNAs, miR-16 and miR-92, were correctly detected on the fully complementary 10-mer PNA probes, mi16–PNA and mi92–PNA, respectively. In contrast, the miRNAs were not detected on the 10-mer DNA probes with the same sequences as the PNAs, and were only weakly detected on the 20-mer DNA probes. The mi16–PNA and mi92–PNA did not bind to nontarget Cy5-labeled miRNAs. When 20 to 50 μM of the PNAs were spotted onto glass slides, the Cy5–miR-16 signals were just visible on the spots of the mi16–mismatch-PNA, which is almost fully complementary to miR-16, but has one mismatched base (Figures 1 and 2). However, the Cy5-miR-16 signal was not detected on the mi16–mismatch-PNA when only 5 μM of the PNAs were spotted (Figure 2). Figure 2 shows that the Cy5–miR-16 signal observed on the 10-mer mi16–PNA was three to four times higher when compared to the 20-mer mi16–DNA-20, and much higher when compared to the corresponding 10-mer DNA probe, while LNA-modified DNA,[11] ANA, and HNA probes[12] were reported as only 2–4 times more sensitive toward RNA than the corresponding DNA probes, although their experimental conditions are different from this study.

A possible drawback of PNAs is their comparatively low water solubility when the chain length is long and/or they contain a purine-rich sequence.[13] However, our results demonstrated that miRNAs could be detected by short 10-mer PNA probes. In addition, highly soluble PNA derivatives, such as OPNA,[14] POPNA,[15] and PAPNA,[16] have been developed. More efficient and highly soluble probes may be prepared by using these PNA derivatives or mixmers of standard PNA and PNA derivatives, which can be synthesized by the Fmoc solid-phase method.[17]

In summary, the sensitivity of PNA arrays toward miRNAs is much higher than with DNA arrays. The detection of mismatched RNAs (with even one mismatch) can be avoided by array preparation using an appropriate concentration (≈5 μM or lower) of PNAs.

We thank Prof. M. Seno and Y. Sugii (Okayama University) for the microarray spotting system, and Y. Sugimoto (Okayama University) for his help. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas "Lifesurveyor" from MEXT and Research for Promoting Technological Seeds from JST to T. O.

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Published on the web (Advance View) March 28, 2009; doi:10.1246/cl.2009.438